after activating relaxes to a lower open probability, but can still be reactivated by a higher $[Ca^{2+}]_i$ (refs 79,80). Whether only one of these is functionally relevant remains controversial, and few cellular studies have addressed this unequivocally. But there is clearly some refractoriness in cellular and local events of SR Ca^{2+} release[44,75]. Recovery of RyR availability occurs with two time constants: one fast (100–300 ms) and one very slow (several seconds). Inactivation of RyRs may be important in minimizing inappropriate SR Ca^{2+} release events between heartbeats. In summary, it seems that both RyR inactivation and partial luminal depletion of SR Ca^{2+} (to reduce RyR opening) both contribute to the turn-off of release. Coupled gating of RyRs (so many gate as one) may also mean that a variant of stochastic attrition also contributes.

Modulation of calcium by sympathetic activation

Physiological sympathetic stimulation of the heart through β-adrenergic receptors increases developed contractions (inotropy) and accelerates relaxation (lusitropy) and $[Ca^{2+}]_i$ decline (Fig. 6). β-Adrenergic receptor stimulation activates a GTP-binding protein (G_S), which stimulates adenylyl cyclase to produce cAMP, which in turn activates PKA. This kinase phosphorylates several proteins related to excitation–contraction coupling (phospholamban, L-type Ca^{2+} channels, RyR, troponin I and myosin binding protein C).

The lusitropic effect of PKA is mediated by phosphorylation of phospholamban and troponin I, which speed up SR Ca^{2+} re-uptake and dissociation of Ca^{2+} from the myofilaments, respectively. But phosphorylation of phospholamban is by far the dominant mechanism for both the lusitropic effect and accelerating the decline in $[Ca^{2+}]_i$ (ref. 81). The faster SR Ca^{2+} uptake also contributes to increasing the SR Ca^{2+} content. The inotropic effect of PKA activation is mediated by the combination of increased I_{Ca} and greater availability of SR Ca^{2+}. This synergistic combination greatly enhances Ca^{2+} transient amplitude, and more than offsets the reduction in myofilament Ca^{2+} sensitivity (caused by troponin I phosphorylation, which by itself would reduce force). The depressant of PKA on the myofilaments seem to be completely attributable to phosphorylation of troponin I (versus myosin-binding protein C), because substitution of troponin I with a non-phosphorylatable troponin I abolishes the myofilament effects of PKA[82].

PKA can also modulate the open probability of RyR channels. In isolated single-channel recordings, PKA increased initial RyR opening during an abrupt $[Ca^{2+}]_i$ rise, but decreased the steady-state open probability at a given $[Ca^{2+}]_i$ (ref. 80). In contrast, Marx *et al.*[33] found that PKA enhanced the steady-state open probability of single RyRs in bilayers, and attributed this to the displacement of FKBP-12.6 from the RyR. Moreover, they found that RyRs were hyperphosphorylated in heart failure, which could cause a diastolic leak of SR Ca^{2+} and contribute to the reduced SR Ca^{2+} content in heart failure (see above). But in more intact cellular systems, no effect of PKA-dependent RyR phosphorylation could be detected on resting SR Ca^{2+} leak (as Ca^{2+} sparks) in the absence of phospholamban (with unchanged SR Ca^{2+} load)[83]. Phosphorylation of RyRs may also alter the intrinsic responsiveness of SR Ca^{2+} release to an I_{Ca} trigger signal, but results concerning this have been mixed, showing an increase, decrease and lack of change[84–86]. Thus, whether PKA-dependent phosphorylation alters RyR behaviour during rest or excitation–contraction coupling remains controversial. This process is particularly challenging to measure in intact cells, because increases in I_{Ca} and in SR Ca^{2+} uptake make isolation of intrinsic RyR effects difficult.

Eisner *et al.*[87] have also argued that, because of autoregulation, altered systolic gating properties of RyRs in intact cells alone exert only transitory effects on Ca^{2+} transient amplitude. That is, abrupt increases in RyR opening or fractional SR Ca^{2+} release cause greater Ca^{2+} extrusion through Na^+/Ca^{2+} exchange at the first beat, thereby decreasing SR Ca^{2+} available for the next beat. In the steady state, this lower SR Ca^{2+} content offsets the increased fractional SR Ca^{2+} release such that Ca^{2+} transients are almost unchanged. However, enhanced diastolic leak of SR Ca^{2+} might still contribute to reduced SR Ca^{2+} load and systolic function in heart failure.

Local signalling is also important in the β-adrenergic receptor cascade. L-type Ca^{2+} channels co-assemble with β_2-adrenergic receptors, G_S, adenylyl cyclase, PKA and phosphatase 2A (at least in brain)[88]. The cardiac RyR serves as both a PKA target and a scaffolding protein (where PKA and phosphatases 1 and 2A are all bound to the RyR through anchoring proteins)[34]. The close physical proximity may be functionally essential[89]. The activation of β_1-adrenergic receptors in ventricular myocytes produces robust inotropic and lusitropic effects, paralleled by phosphorylation of Ca^{2+} channels,

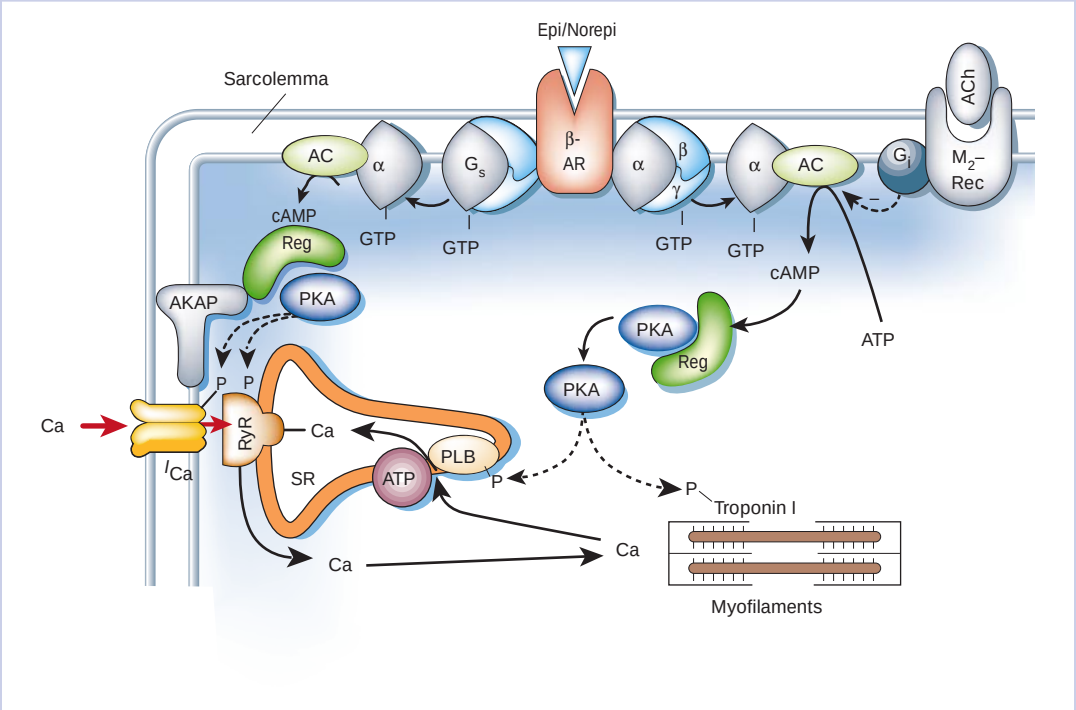

Figure 6 β-Adrenergic receptor activation and phosphorylation targets relevant to excitation–contraction coupling. AC, adenylyl cyclase; ACh, acetylcholine; AKAP, A kinase anchoring protein; β-AR, β-adrenergic receptor; M_2-Rec, M_2-muscarinic receptor; PLB, phospholamban; Reg, PKA regulatory subunit; SR, sarcoplasmic reticulum.

phospholamban and troponin I. By contrast, the activation of β_2-adrenergic receptors may be more restricted to I_{Ca} enhancement[90], and β_2-adrenergic receptors are located almost exclusively in specialized sarcolemmal invaginations called caveolae (versus β_1-adrenergic receptors, which are largely non-caveolar)[91].

Activation of other G-protein-coupled receptors that greatly stimulate cAMP production (for example, prostaglandin E, histamine, glucagon-like peptide-1) produce little or no inotropic effect (as compared with β_1-adrenergic receptors)[92]. Thus, the functionally important levels of cAMP, activated PKA, phosphatase and phosphodiesterase (which breaks down cAMP) are those very near to that of the target protein. The total cellular concentration of cAMP might be irrelevant to key regulatory pathways, except as an overflow from local cAMP-mediated signal transduction. However, if this is true, it is less clear how targeting would practically work for phospholamban and troponin I phosphorylation (as compared with I_{Ca} or RyR). This would require very high amounts of the various anchoring and signalling proteins, because troponin I and phospholamban are present at 50 μM or higher concentrations and are dispersed widely in the cell.

Relative receptor locations can also regulate this signalling cascade. For example, M_2-muscarinic receptor activation can either decrease or increase concentrations of cAMP levels, depending on whether they were produced by β_1- or β_2-adrenergic receptors, respectively[93]. This may be due in part to the relative exclusion of M_2-muscarinic receptors from caveolae. Thus, the location of receptors and their signalling cascade components can selectively determine function.

Implications for calcium handling

Calcium in cardiac myocyte is in a dynamic yet delicate balance, created by multiple interacting cellular systems that can be tuned by physiological modulators. It is also clear that we must think increasingly in terms of microdomains and local control, without losing perspective on the integrative framework in which these domains function. RyR and I_{Ca} are both responsible for, but also controlled by, the local cleft $[Ca^{2+}]_i$ (which may differ greatly from the $[Ca^{2+}]_{sm}$ that controls Na^+/Ca^{2+} exchange). Key regulatory pathways (for example, β-adrenergic receptors, calmodulin and possibly Ca^{2+}-dependent transcription) also exhibit local functional coupling in microdomains. These various signalling domains surely overlap spatially and functionally.

It will be important to develop new experimental tools to assess how the key signalling molecules (including Ca^{2+}) interact functionally and are targeted to the appropriate microdomains. Future studies will need to clarify how cells distinguish between Ca^{2+} involved in excitation–contraction coupling and transcriptional regulation. We may also have to start thinking more stochastically about local reactions. For example, at resting $[Ca^{2+}]_i$ there is less than one free Ca^{2+} ion in an entire junctional cleft, making the concept of collision probability more meaningful than concentration. Thus, much challenging work lies ahead if we are to understand the physiological functions of many of these processes *in situ*, particularly with respect to signalling in microdomains, as well as the pathophysiological and therapeutic implications. □

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